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Interactions with LC3 and polyubiquitin chains link nbr1 to autophagic protein turnover

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ABSTRACT

Nbr1, a ubiquitous kinase scaffold protein, contains a PB1, and a ubiquitin-associated (UBA) domain. We show here that the nbr1 UBA domain binds to lysine-48 and -63 linked polyubiquitin-B chains. Nbr1 also binds to the autophagic effector protein LC3-A via a novel binding site. Ubiquitin-binding, but not PB1-mediated p62/SQSTM1 interaction, is required to target nbr1 to LC3 and polyubiquitin-positive bodies. Nbr1 binds additionally to proteins implicated in ubiquitin-mediated protein turnover and vesicle trafficking: ubiquitin-specific peptidases USP8, and the endosomal transport regulator p14/Robld3. Nbr1 thus contributes to specific steps in protein turnover regulation disrupted in several hereditary human diseases.

Structured summary:
MINT-7034452: *USP8* (uniprotkb:P40818) *physically interacts* (MI:0218) with *NBR1* (uniprotkb:Q14596) by *pull down* (MI:0096)
MINT-7034438: *SQSTM1* (uniprotkb:Q13501) and *LC3* (uniprotkb:Q9H492) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7034309: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *pull down* (MI:0096)
MINT-7034323: *NBR1* (uniprotkb:P97432) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *pull down* (MI:0096)
MINT-7034233: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *USP8* (uniprotkb:P40818) by *two hybrid* (MI:0018)
MINT-7034207: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *Robld3* (uniprotkb:Q9JHS3) by *two hybrid* (MI:0018)
MINT-7034400, MINT-7034418: *NBR1* (uniprotkb:Q14596) and *LC3* (uniprotkb:Q9H492) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7034167: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *Ubiquitin B* (uniprotkb:Q78XY9) by *two hybrid* (MI:0018)
MINT-7034470: *NBR1* (uniprotkb:Q14596) and *USP8* (uniprotkb:P40818) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7034194: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *LC3-A* (uniprotkb:Q91VR7) by *two hybrid* (MI:0018)
MINT-7034336: *SQSTM1* (uniprotkb:Q13501) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *pull down* (MI:0096)
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MINT-7034350: *NBR1* (uniprotkb:Q14596) and *Ubiquitin* (uniprotkb:P62988) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7034181: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *Tmed10* (uniprotkb:Q9D1D4) by *two hybrid* (MI:0018)
MINT-7034220: *NBR1* (uniprotkb:Q14596) *physically interacts* (MI:0218) with *ube2o* (uniprotkb:Q6ZPJ3) by *two hybrid* (MI:0018)

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1. Introduction

Cellular protein turnover can use the ubiquitin–proteasome system (UPS) or autophagy, two degradation pathways that share the modification of target proteins by small modifier proteins in sequential enzymatic cascades, and their subsequent sequestration to specific degradation machineries. The UPS targets mostly soluble, cytosolic proteins that are modified by ubiquitin and then recruited to, and destroyed by, the proteasomal proteases. Autophagy is the mechanism by which damaged organelles and both soluble and aggregated proteins can be removed (reviewed in [1,2]). The prerequisite of initial "labelling" of target proteins by ubiquitin via specific ligases, their subsequent recognition by adapter proteins, and the ensuing recruitment to the destruction machinery in proteasomes and autophagosomes mediates certain elements of specificity.

In autophagy, target proteins are recruited to and enveloped by phagophore membrane structures containing the specific lipidated ubiquitin-like protein ATG8/LC3 [3]. These then fuse with lysosomes to form autolysosomes, leading to the enzymatic destruction of their cargo. Autophagy is important for the continuous ordered remodelling of tissues during postnatal development and physiological adaptation, and not only during extreme conditions like nutrient starvation [1].

One of the multifunctional adaptor proteins that link cellular growth and survival signals to protein degradation via the proteasome and autophagy is p62/SQSTM1 (also known as ORCA, lckBP, A170 [4,5]) that has recently been implicated in regulating the autophagy of ubiquitinated proteins [6] by its direct interaction with LC3 [7–9]. p62/SQSTM1 interacts with, and shares many structural features with the 107 kDa protein nbr1 [10], a protein highly conserved between human (*NBR1* gene) and mouse (*Nbr1* gene). Both proteins contain an N-terminal p67phox/ Bem1p (PB1) domain (also referred to as ubiquitin-like domain [5]), a ZZ Zinc finger domain, and a C-terminal UBA domain (Fig. 1). Whereas the type-A/B PB1 domain of p62/SQSTM1 allows the formation of oligomeric complexes both with p62/SQSTM1 and other PB1 domain proteins [4], nbr1 contains a type-A PB1 domain [11] and only forms heterodimeric interactions through this motif; but homodimerises via the N-terminal of two coiled-coil domains. P62/SQSTM1 is currently the only known nbr1 PB1 ligand interacting directly in a canonical PB1–PB1 fashion [12]. In addition, the PB1 region of nbr1 also binds to the open catalytic site of the giant muscle protein kinase titin [13]. Other links of nbr1 to protein kinase signaling exist through the atypical PKC adaptor protein FEZ1 [14] (Fig. 1). A mutation in human titin kinase that weakens the nbr1 interaction leads to a severe hereditary myopathy, human myopathy with early respiratory failure (HMERF) with disrupted myofibrils, localization of nbr1 to cytoplasmic bodies and accumulation of p62/SQSTM1 with aggregated myofibrillar proteins [13], suggesting a defect in proteasomal or autophagic turnover. In contrast to p62/SQSTM1 however, very little is known about the ligands of nbr1 mediating the potential involvement in ubiquitin-mediated protein turnover that might be inferred from its domain pattern. To understand this ubiquitously expressed protein in better detail, we performed interaction analysis in bone and muscle yeast two-hybrid screens, and found generally expressed ligands that link nbr1 to ubiquitin- and LC3-mediated protein turnover.

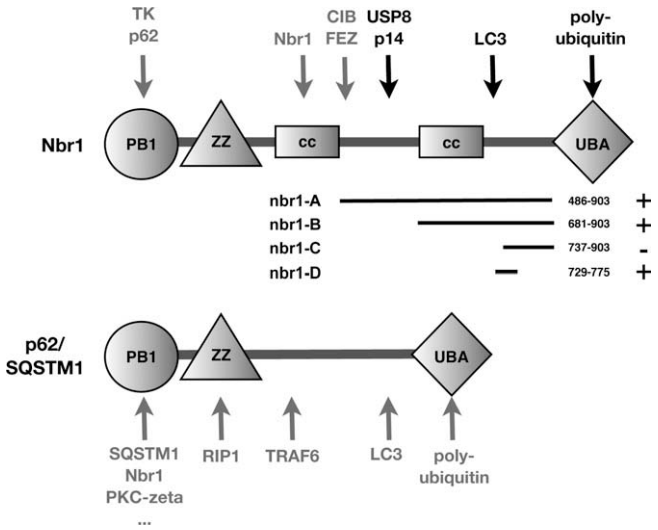

Fig. 1. Schematic layout of the domain organisation of nbr1 and p62/SQSTM1 and the sites of protein–protein interactions; known nbr1 ligands are shown in grey. The PB1 region was shown to bind p62/SQSTM1 and the open conformation of the giant muscle protein kinase titin [13]; the middle segment binds fasciculation and elongation protein zeta-1 (FEZ1), a PKCzeta interacting protein, and calcium and integrin binding protein (CIB) [14]. The new nbr1 interactions described here are shown in black. The extent of truncation constructs of nbr1 used for GST-binding assays is shown. LC3 interactions are shown as + for positive and − for negative. PB1, Phox/Bem1 homology 1 domain; ZZ, ZZ zinc finger domain; cc, coiled-coil domain; UBA, ubiquitin associated domain.

2. Materials and methods

2.1. Nbr1 constructs and yeast two hybrid screening

Baits from the human *NBR1* or the mouse *Nbr1* gene were amplified by PCR and cloned into the required vectors; the extent of the constructs is shown in Fig. 1. Yeast two-hybrid screening was essentially performed as described previously [15]. pLex were co-transformed with a pGAD10 human cardiac muscle (Clontech) or mouse calvarial bone cDNA library (a kind gift from Prof. Ikramuddin Aukhil, University of Florida) into the L40 yeast strain by standard procedures. Transformants were screened by HIS3 reporter gene activity, and selected clones were analysed for β-galactosidase reporter gene activity.

2.2. GST pulldown assays

GST pulldown assays were essentially performed as described [16]. COS7 cells were transfected with HA-tag or pEGFP-fusion constructs and extracted with IP buffer (10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.5% NP-40, 1 mM DTT supplemented with a protease inhibitor cocktail (Roche)) and the cell lysates were incubated with beads coupled with the appropriate GST fusion proteins, or GST alone, for 2 h on ice. Following incubation, beads were washed four times in IP buffer. The proteins retained on the beads were separated on SDS–PAGE and then transferred on nitrocellulose membrane following standard procedures. Blots were treated with mouse monoclonal anti-GFP or rat monoclonal anti-HA tag (Roche) and subsequently with a secondary antibody (HRP-conjugated anti-mouse or anti-rat (DAKO Cytomation, Glostrup, Denmark). Detection was performed by ECLTM Western blotting analysis.

2.3. Ubiquitin binding assays

Two micrograms of GST fusion protein bound to glutathione beads was incubated with 2 μg of K48 (Boston Biochem UC-230) or K63 (Boston Biochem UC330) ubiquitin chains ranging from two to seven and 400 μl IP buffer and were processed as above. Samples were analysed by SDS–PAGE and Western blotting using a rabbit polyclonal antibody against ubiquitin (Dako, Z0458). Myc-tagged ubiquitin mutants used for cellular colocalisation studies were a kind gift from Dr. Jo Morris, KCL.

2.4. Bacterial expression of fusion proteins

Human NBR1 (nbr1-A 486–903aa, nbr1-2 681–903 aa, nbr1-C 737–903 aa and nbr1-D 729–775 aa) and USP8 were cloned into the pGST2C vector using the XhoI–BamHI sites and soluble proteins were expressed in BL21[DE3] cells by standard protocols. The fusion proteins were purified by glutathione affinity chromatography using standard procedures. The YFP-LC3 plasmid was a kind gift by Dr. Marco Sandri. LC3 was expressed in fusion with a His6-TEV site tag using a modified pET expression plasmid [17].

2.5. Cell culture, transfection and immunostaining

COS7 cells were cultured in DMEM/10% fetal bovine serum by standard protocols and transfected using Fugene 6 (Roche). Cells were lysed after 24 h in 500 μl IP buffer for pulldown assays, or were fixed with 4% paraformaldehyde in PBS for 6 min, then processed for immunostaining as described [17]. All fluorescent-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (USA) [18].

2.6. Primary antibodies

The following primary antibodies were used. Mouse monoclonal anti-Nbr1 (Abcam, UK); mouse monoclonal anti-conjugated ubiquitin FK2 (Biomol) and rabbit polyclonal anti-ubiquitin (Dako, UK); rabbit polyclonal anti-LC3 (Cell Signaling Technology/New England Biolabs, UK); rat monoclonal anti-HA tag (Roche); and rabbit polyclonal anti-Myc tag A14 (Santa Cruz).

2.7. Confocal microscopy and image analysis

Cell preparations were imaged with a Zeiss LSM510 confocal microscope in sequential scanning mode, using a 63× oil immersion objective and instrument zoom rates between 1× and 2.5×.

3. Results

3.1. Multiple interactions link nbr1 to protein turnover regulation

For the identification of new nbr1 ligands, yeast two-hybrid screens with full-length nbr1, the nbr1 UBA domain, and two fragments lacking both the PB1 and UBA domains (residues 93–903 and 495–924) were performed in mouse or human bone and muscle cDNA libraries essentially as described [17]. Several hundred HIS3 and β-galactosidase positive clones were isolated, and reporter gene activation was tested to be bait-dependent. The inserts of clones showing bait-dependent HIS3/β-GAL activation were sequenced and analysed. Here, we report only those clones that are not tissue-specific; bone- and muscle-specific ligands will be described elsewhere.

Most clones interacting with full-length or C-terminal nbr1 encoded ubiquitin-B (Table 1), but few ubiquitin A and C clones were also identified. The interaction with ubiquitin was mapped by truncation mutants to the UBA domain. Links to ubiquitin protein modification were also found by interactions with the ubiquitin conjugating enzyme Ube2o, and ubiquitin-specific peptidase USP8 (UBPY) (Table 1). Additionally, we identified microtubule associated protein light chain-3 (LC3, the mammalian homologue of yeast ATG8) as an nbr1 ligand. A link to vesicular trafficking was identified by the interaction with p14/Robld3 [19]) and transmembrane enp24-like trafficking protein 10.

3.2. The nbr1 UBA domain binds both lysine-48 and lysine-63 linked polyubiquitin chains

As the interaction of nbr1 with ubiquitin identified in living yeast will be complemented by the yeast ubiquitination machinery, and could be directed towards monoubiquitin or various polyubiquitin chains, we characterised the nature of this interaction using glutathione-S-transferase (GST) tagged proteins *in vitro*. The GST-tagged nbr1 UBA domain was tested for its ability to interact with lysine-48 and -63 (K48 or K63) linked polyubiquitin chains in pulldown assays using synthetic polyubiquitin chains, and compared to the binding properties of p62/SQSTM1 UBA. We observed comparable binding to both types of ubiquitin chains (Fig. 2A), although affinity of p62/SQSTM1 for K63 linked chains seems weaker. This ability to bind both common types of polyubiquitin chains is shared with the structural homologue p62/SQSTM1 [20], although the affinity of nbr1 seems to be higher for K63 chains in comparison to p62/SQSTM1, which in this assay favours K48-linked ubiquitin. In agreement with these *in vitro* data, nbr1 colocalised in cytoplasmic bodies with conjugated ubiquitin detected by the FK2 antibody (Fig. 2B), and with ubiquitin mutants abolishing all but lysine-63 (Fig. 2B) and lysine-48 (not shown) conjugation. However, colocalisation with a mutant ubiquitin abolishing K63, K48 and K33 conjugation almost completely abolished colocalisation of nbr1 in ubiquitin-positive bodies (Fig. 2B).

3.3. Nbr1 binds LC3 at a distinct site

The interaction with LC3 suggests an involvement of nbr1 in autophagic protein turnover, similar to the structurally related p62/SQSTM1. However, the recently identified LC3 binding site in p62/SQSTM1 [7,21] shows no easily identified homology to nbr1. We therefore created a series of GST-tagged truncation fragments of nbr1, nbr1-A to nbr1-D (Fig. 1), to map the LC3 interaction in pulldown binding assays using YFP-tagged LC3. These were also tested with bacterially expressed LC3 to rule out the participation of cellular factors in the pulldown assays. Nbr1 fragments lacking the PB1, ZZ, coiled-coil-1 or UBA domains retained their ability to interact with LC3 (Figs. 1 and 3A). Truncation of the coiled-coil domain 2 (aa 708–903) did not affect LC3 interaction (not shown), while further truncation of an acidic-hydrophobic-rich segment just C-terminal to coiled-coil 2 up to the first proline residue after the predicted helical coiled-coil 2 segment (nbr1-C) abolished LC3 binding. A 46-residue construct starting just at the end of coiled-coil2 (nbr1-D, 729–775) bound strongly to LC3. The difference between the overlapping constructs nbr1-C and nbr1-D resides in the eight amino acids SEDYIIIL, showing that these residues are crucial

Table 1
Ubiquitously expressed ligands of nbr1 identified in yeast two-hybrid screens from human muscle and mouse bone cDNA libraries.

Protein	Accession number	Clones	Region
Ubiquitin-B	NM_011664.3	12	Full length
LC3-A	NM_025735.1	3	18–end
Ubiquitin-specific peptidase USP8 (UBPY)	NM_005154	2	Full length
Ubiquitin conjugating enzyme E2O (Ube2o)	NM_173755.3	2	1164–1288
p14, Robld3	NM_031248.3	1	Full length
Transmembrane enp24-like trafficking protein 10	NM_026775.4	1	78–end

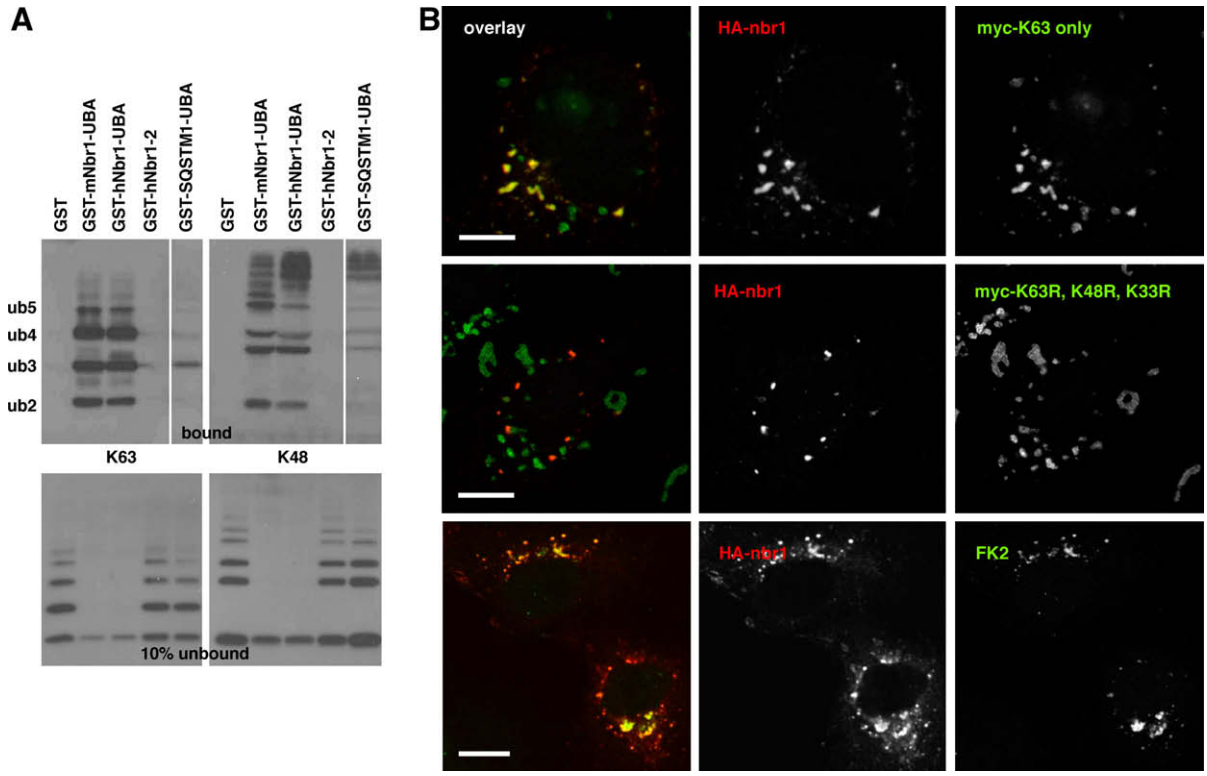

Fig. 2. Nbr1 binding to polyubiquitin chains. (A) GST-fused human (hNbr1) and mouse (mNbr1) UBA domains were immobilised on glutathione beads and incubated with K48 and K63-linked polyubiquitin chains. An N-terminal fragment of human nbr1 without the UBA domain (hNbr1-2) and GST were used as negative controls; the UBA domain of p62/SQSTM1 served as positive control. Bound ubiquitin was detected by Western blotting with anti-ubiquitin antibodies. (B) Transfected human nbr1 in COS7 cells colocalised with wild-type polyubiquitin chains detected by the FK2 monoclonal antibody, as well as with mutant ubiquitin in which all known lysine modification sites have been replaced by arginine except for K63 (myc-K63 only), while colocalisation with mutant ubiquitin in which lysines 33, 48 and 63 have been replaced with arginine (myc-K63R, K48R and K33R) almost completely abolished colocalisation. Transfected nbr1 was detected via its N-terminal HA-tag, the mutant ubiquitins via their N-terminal myc-tag. Scale bars: 10 μm.

for LC3 binding. This segment is in a similar position as the LC3 binding site in p62/SQSTM1 but lacks an obvious consensus sequence with p62/SQSTM1.

3.4. The UBA domain is necessary to recruit nbr1 to LC3-positive bodies

We assessed the localization of endogenous nbr1 and LC3 in COS7 cells treated for 4 h with bafilomycin-A_1, a specific cellular inhibitor of vacuolar type H(+)-ATPase that inhibits the acidification of organelles containing this enzyme and leads to accumulation of unprocessed autophagosomes [22]. In bafilomycin-treated cells, endogenous LC3 and nbr1 colocalise in cytoplasmic bodies, whereas in untreated cells, both proteins are weakly stained and sporadically colocalise (Fig. 3B). When we co-transfected HA-tagged nbr1 with YFP-LC3 in COS7 cells, we observed nbr1 accumulation in larger vesicle-like bodies that are very similar to those observed for p62/SQSTM1 and LC3 (Fig. 3C). We suspected that these reflect components of the autophagic pathway due to the association with ubiquitin and LC3. Co-localization of nbr1 with LC3 was independent of the presence of the PB1 domain that forms the interface with p62/SQSTM1 [12,13], suggesting that nbr1 is not indirectly recruited to LC3-positive bodies via p62/SQSTM1. Indeed, p62/SQSTM1 and truncated nbr1 lacking the PB1 domain both localize to cytoplasmic bodies but do not colocalise (Fig. 3C), indicating that the ubiquitin and LC3-dependent targeting of nbr1 is independent of p62/SQSTM1 and vice versa. However, deletion of the UBA domain completely abrogated nbr1 recruitment to LC3 positive bodies, and both proteins were found to be diffusely colocalised in the cytoplasm (Fig. 3C).

3.5. Nbr1 and USP8/UBPY interact and colocalise

The nbr1 ligand ubiquitin-specific protease USP8/UBPY was expressed as a GST fusion protein and confirmed for its ability to interact with HA-tagged nbr1 *in vitro* (Fig. 4A), while we found that an unrelated component of the ubiquitination machinery, UBE2E, did not interact. As USP8 has been described as an endosomal ubiquitin peptidase, we compared the cellular localization of nbr1 and USP8 in COS7 cells. Under these conditions, we find that transfected nbr1 and USP8 colocalise in cytoplasmic bodies (arrowheads in Fig. 4B).

4. Discussion

We have identified a number of new ligands of nbr1 that links this kinase scaffold to protein turnover via autophagy and proteasomal degradation. The nbr1 UBA domain binds to ubiquitin-B, and more specifically to K48 and K63-linked polyubiquitin chains, and thus behaves similar to the related p62/SQSTM1 [20]. However, the UBA domains of these two proteins are only 32% identical, underscoring divergent ligand specificities that may also involve ubiquitin-independent interactions like the interaction with MuRF1/2 of p62/SQSTM1 [13]. This may explain why, despite overall similarity between both proteins, their intracellular traffic seems to be different, as shown by the distinct localization of p62/SQSTM1 and the PB1-deleted form of nbr1 (Fig. 3C). It is noteworthy that the affinity of nbr1 for K63-linked polyubiquitin appears to be higher than that of p62/SQSTM1. Our results demonstrate also that nbr1 is able to recruit ubiquitinated proteins

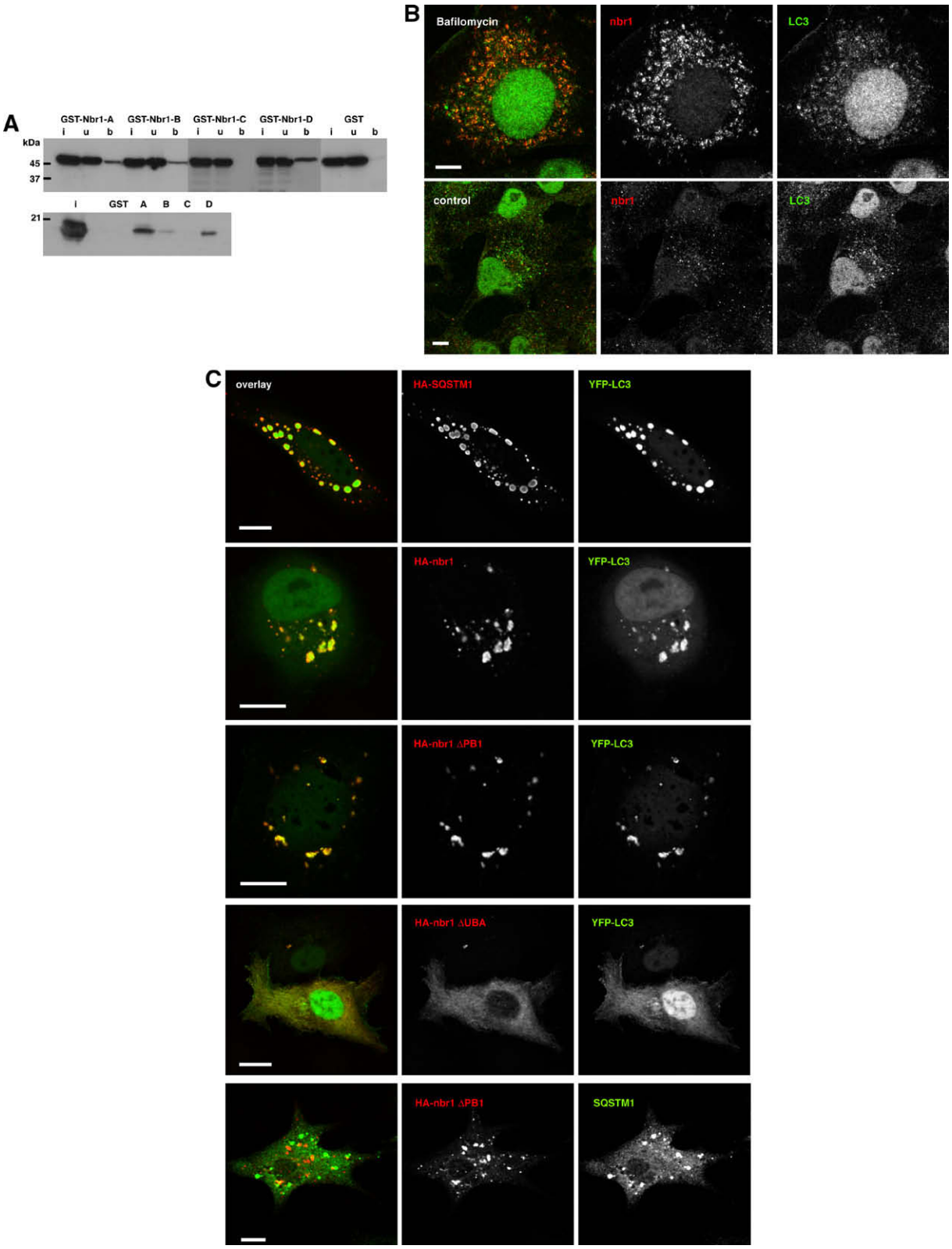

Fig. 3. Nbr1 interacts with LC3. (A) GST-fused human nbr1 constructs phased as in Fig. 1 were bound to glutathione beads and incubated with cell lysates from YFP-LC3 transfected COS7 cells or recombinant LC3. Upper panel: results for YFP-LC3. Lower panel: similar results were obtained using the same GST-nbr1 fragments (abbreviated as A to D) and bacterially expressed His6-LC3, detected in Western blot by the LC3 monoclonal antibody (bound fractions shown only). i, input; u, unbound; b, bound fractions. (B) Endogenous nbr1 and LC3 colocalise in COS7 cells treated for 4 h with bafilomycin-A (top panel), whereas levels are low when autophagic flux is not inhibited (bottom panel). (C) Colocalisation of transfected human nbr1 and LC3 in COS7 cells. LC3 colocalises in presumptive autophagosomes with the known ligand p62/SQSTM1, as well as with full-length nbr1 (HA-nbr1) and with nbr1 lacking the PB1 domain (HA-nbr1ΔPB1). Deletion of the UBA domain (HA-nbr1ΔUBA) abolished the formation of LC3-positive bodies, and both proteins were more diffusely colocalised in the cytoplasm. Deletion of the PB1 domain (HA-nbr1ΔPB1) allowed the formation of LC3 positive bodies but abrogated the colocalisation with p62/SQSTM1. Scale bars: 10 μm.

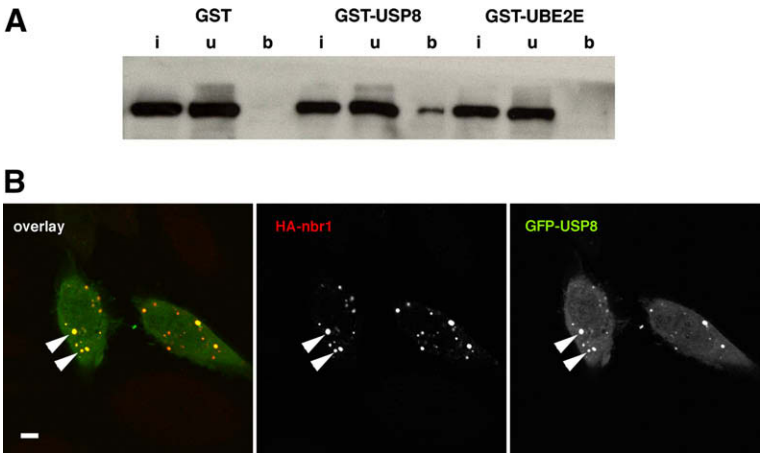

Fig. 4. Nbr1 interacts with USP8. (A) GST-fused human USP8 or UBE2E were bound to glutathione beads and incubated with cell lysates from HA-nbr1 transfected COS7 cells. Nbr1 only interacts with USP8, not with the control proteins GST and UBE2E. i, input; u, unbound; b, bound fractions. (B) Colocalisation of human HA-tagged nbr1 and GFP-tagged USP8 in cytoplasmic bodies of transfected COS7 cells. Arrowheads: colocalised nbr1 and USP8. Scale bar: 10 μm.

to LC3-positive bodies independently of p62/SQSTM1 and may thus act redundantly to many of the latter's functions. This possible redundancy may have functional consequences when analysing genetic defects or models of both proteins. For instance, it may explain why the mutations in the p62/SQSTM1 UBA domain that cause Paget's disease of the bone do not appear to elicit a more generalised phenotype [23]. Furthermore, knockout animals of p62/SQSTM1 have a tissue-restricted phenotype [9,24], arguing again that some functions of p62/SQSTM1 can be taken over by functionally analogous proteins like nbr1.

Overlapping functions of nbr1 and p62/SQSTM1 are also suggested by their shared ability to bind to LC3 and polyubiquitin simultaneously, thus presumably acting to recruit ubiquitinated proteins to autophagosomes. The LC3 binding region of nbr1, however, shows no clear sequence homology to the structurally related p62/SQSTM1. This sequence of ca. 8 residues is in a position N-terminal to the UBA domain, an arrangement analogous to the LC3 binding site in p62/SQSTM1 (Fig. 1). However, sequence homology in this region is low, although reminiscent of the LC3 binding site in p62/SQSTM1. These results are in excellent agreement with those published by Kirkin et al. [25] while this work was reviewed. It is not clear whether the LC3 binding site in p62/SQSTM1 [7,8,21] suffices for autophagosome recruitment. Interestingly however, LC3 interaction is insufficient for nbr1 to induce formation of large autophagosomes-like structures, which requires the presence of the UBA domain (Fig. 3C). Although we show that LC3 can bind to nbr1 constructs in the absence of coiled-coil 1 and 2, the dimeric nature of nbr1 via its coiled-coil domains may be important to cluster LC3-positive phagophore membranes around polyubiquitinated target proteins during stages of autophagosomes maturation [3]. The large bodies formed upon overexpression of nbr1, but also of p62/SQSTM1, are obviously artifactual structures that suggest that overexpression of nbr1 and p62 blocks the accurate processing of autophagosomes. This effect may be useful to trap and identify cellular targets for autophagosomal degradation delivered via these adaptor proteins, which are expected to accumulate in the LC3-positive bodies.

The interaction of nbr1 with the ubiquitin-specific peptidase USP8/UBPY points further to a role of nbr1 in regulating the dynamic traffic and fate of ubiquitinated proteins in endosomes, lysosomes and autophagosomes. USP8 can process K48- and K63-linked polyubiquitin chains, and localizes to endosomes, which accumulate ubiquitinated proteins [26,27]. The interaction with nbr1, however, appears to be independent of polyubiquitin chains, and the function of nbr1 therefore seems to be that of a scaffold that targets modulators of ubiquitin signalling to vesicular structures accumulating polyubiquitinated proteins at LC3-labelled phagophores.

In summary, our results show that the protein kinase-associated scaffold protein nbr1 binds to several proteins implicated in the traffic of ubiquitinated proteins to lysosomes and autophagosomes. Further links to vesicle traffic were found (Table 1). Disruption of nbr1 function may therefore contribute to several human diseases where protein turnover is defective. In muscle, a mutation in the protein kinase domain of the nbr1 ligand titin abrogates nbr1 binding and leads to a severe myopathy with early respiratory failure [13]. Our results now implicate the failure of proper protein homeostasis via autophagy and proteasomal turnover in the mechanism of this disease, might explain the occurrence of nbr1-positive vesicle-like bodies in the diseased muscles [13], and would agree well with the increasingly recognised role of autophagy in muscle mass regulation [28]. The link of both p62/SQSTM1 and nbr1 to ubiquitin-mediated autophagy also raises the interesting possibility that nbr1 might be an unrecognised player in other genetic conditions. p62/SQSTM1 is the most common gene mutated in patients with Paget's disease (reviewed in [23]). Similarly, mutations in valosin-containing protein (VCP), which is involved in flux regulation of ubiquitinated proteins in proteasomes and lysosomes, cause a complex combination of myopathy, Paget's disease and frontotemporal dementia (reviewed in [29]). The partly overlapping functions of nbr1 and p62/SQSTM1 suggest that similar functions in bone and muscle remodelling might be implicated in defects leading to human bone and muscle disease. Physiological studies of nbr1 function in specific tissues will now need to build upon the generic nbr1 interactions identified here.

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